Circulating tumor cells (CTCs) in Ovarian Cancer

In our early studies, we used **Oncoquick® for CTC enrichment**, a density gradient centrifugation device which allowed to use the complete supernatant above the porous barrier. After some washing steps, cells were spun onto glass slides and analyzed for **CK-positive cells** as already described for the detection of DTCs. A complete monitoring of CK+ cells in blood samples before and after chemotherapy could be performed for 45 patients (**Figure 1; Wimberger et al., 2007**).

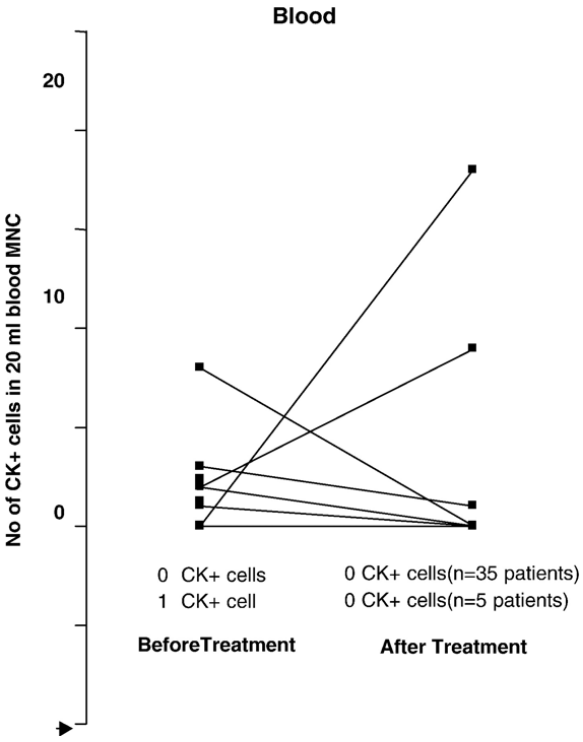

Figure 1: Monitoring of CK+ cells in blood before and after platinum-based chemotherapy. ■ Indicates the number of CK+ cells per 20 ml blood MNC. Abbreviations: CK: cytokeratin; MNC: mononuclear cells (***Wimberger et al., 2007***).

Since **detection rates** were **not convincing** and **characterization** of **CTCs** was hardly feasible, we continued with the **AdnaTest**, already described for our BC studies. Briefly, CTCs were captured with immunomagnetic beads targeting EpCAM (GA 73.3) and MUC1 (***AdnaTest BreastCancerSelect***), followed by the analysis of HER2, MUC1, CA-125 and GA 733-2 transcripts using RT-PCR. A sample was considered positive for CTCs, if one of the transcripts was expressed above the threshold concentration. Applying this method, we analyzed 122 OC patients before surgical intervention and/or after chemotherapy and detected **CTCs** in **19%** of the patients before and in **27% after therapy** which significantly correlated with a **shorter OS** but not with PFS, which was explained by incomplete tumor resection in almost half of the patients **(Figure 2; Aktas et al., 2011**).

Some years later, we investigated the analysis of ***EpCAM***, ***MUC1***, ***MUC16*** *and* ***ERCC1*** *(*excision repair cross-complementation group 1) transcripts in 143 OC patients at primary diagnosis using the same selection procedure as published in 2011. An **overall CTC-positivity** rate of **14%** significantly correlated with a **reduced OS** and a multivariate analysis confirmed the presence of **ERCC1-positive CTCs** to be significantly associated with **a reduced PFS, OS** and a **clinical platinum resistance**, whereas immunohistochemical staining for ERCC1 on the **primary tumor** tissue samples did not achieve **any significant association** (**Figure 3; Kuhlmann et al., 2014**).

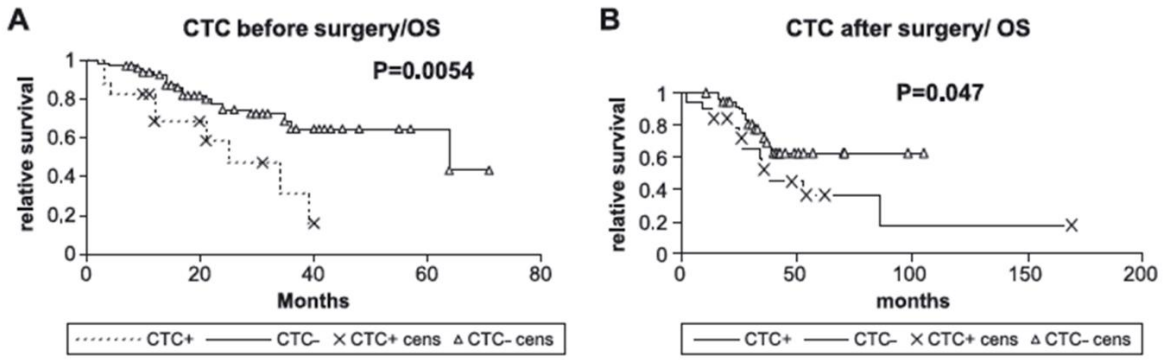

Figure 2: Prognostic significance of CTCs before and after therapy with regard to OS (***Aktas et al., 2011***).

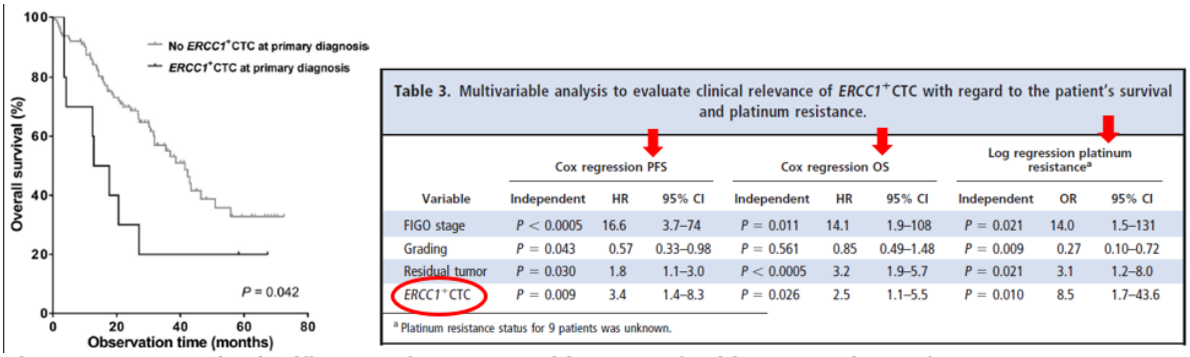

Table 3. Multivariable analysis to evaluate clinical relevance of *ERCC1*⁺CTC with regard to the patient's survival and platinum resistance.

	Cox regression PFS			Cox regression OS			Log regression platinum resistance[a]		
Variable	Independent	HR	95% CI	Independent	HR	95% CI	Independent	OR	95% CI
FIGO stage	$P < 0.0005$	16.6	3.7–74	$P = 0.011$	14.1	1.9–108	$P = 0.021$	14.0	1.5–131
Grading	$P = 0.043$	0.57	0.33–0.98	$P = 0.561$	0.85	0.49–1.48	$P = 0.009$	0.27	0.10–0.72
Residual tumor	$P = 0.030$	1.8	1.1–3.0	$P < 0.0005$	3.2	1.9–5.7	$P = 0.021$	3.1	1.2–8.0
ERCC1⁺CTC	$P = 0.009$	3.4	1.4–8.3	$P = 0.026$	2.5	1.1–5.5	$P = 0.010$	8.5	1.7–43.6

[a] Platinum resistance status for 9 patients was unknown.

Figure 3: Prognostic significance of ERCC1-positive CTCs (***Kuhlmann et al., 2014***).

In a follow-up study, the analysis 65 **paired pre- and post-chemotherapeutic** OC blood samples, further confirmed the **prognostic relevance** of **present and persistent *ERCC1*** transcripts in CTCs with regard to a **poor PFS and OS**. Using ***ERCC1* transcripts** as an additional identification marker for CTCs, independent of *EpCAM*, *MUC1* or *CA-125* positivity, the **CTC detection rate expanded** from 23% to **40% before** surgery and from 20% to **38% after chemotherapy**, respectively. In addition, the presence of *ERCC1* transcripts in combination with transcripts for *EpCAM*, *MUC1* or *CA-125* after therapy, showed a reduced PFS and OS, while ***ERCC1*-positivity** correlated with **platinum-resistance** (**Chebouti et al., 2016**).

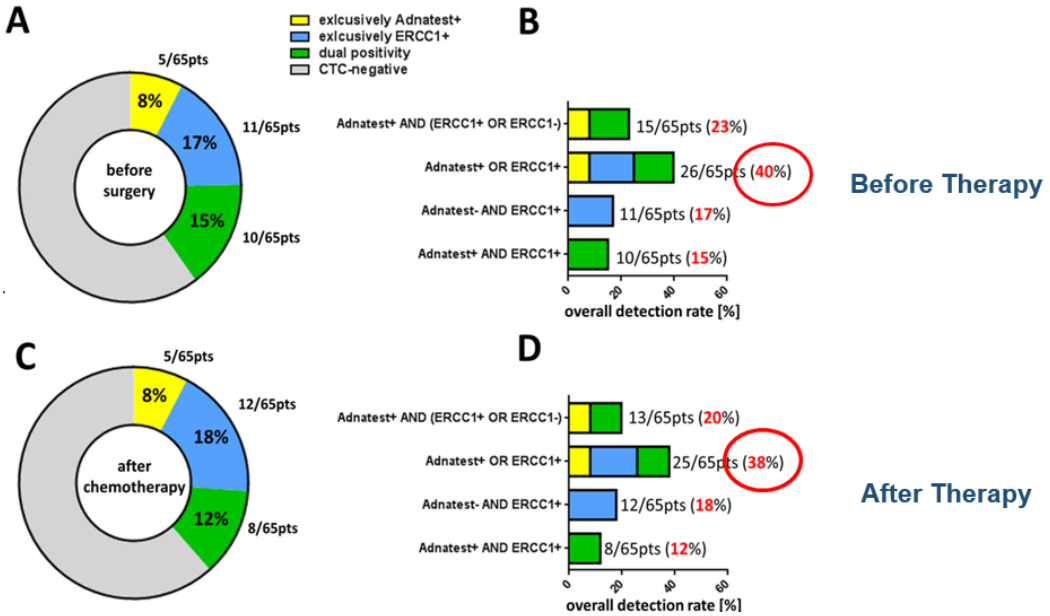

Figure 4: Inclusion of *ERCC1* in the AdnaTest OvarianCancer significantly enhances the CTC-detection rate (***Chebouti et al., 2016***).

Chebouti et al. expanded their research and included the **detection of mesenchymal-like CTCs** in OC patients. Using immunomagnetic selection followed by multi-marker RT-PCR for the detection of epithelial CTCs and **CTCs in EMT** (*PI3Kα*, *AKT-2* and *TWIST*) in 91 OC patients **before therapy**, **24%** of the detected **CTCs** were **epithelial** and **58% EMT-like**. After therapy, the latter subtype **increased to 76%** whereas epithelial CTCs were only present in 12% of the patients. Furthermore, a dual positivity for epithelial and EMT-like transcripts was only found in a minor percentage of analyzed cases. Interestingly, besides therapeutic selection of EMT-like CTCs under platinum-based chemotherapy, **double-positive CTCs** expressing ***PI3Kα*** and ***TWIST***, were only present **after therapy**, reflecting tumor evolution in response to the given chemotherapy. Moreover, the presence of **epithelial CTCs in combination** with the detection of ***PI3Kα*** transcripts indicated a **poor prognosis** at the time of primary diagnosis (**Figure 5; Chebouti et al., 2017**).

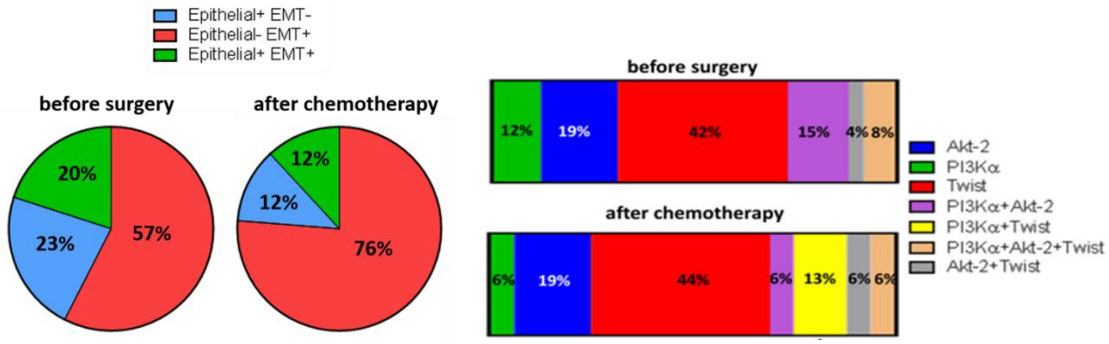

Figure 5: EMT-like circulating tumor cells in OC patients are enriched by platinum-based chemotherapy (***Chebouti et al., 2017***).

Currently, approaches for **single CTC (sCTC) analysis** are under way to gain more information on the clonal evolution of CTCs and their impact on disease progression and/or treatment resistance.

In a **proof of principle study** we recently presented a workflow (**Figure 6**) to generate **sCTC genomic data**, with the need of further studies to improve the CTC detection rate and enable insights into tumor evolution on a sCTC resolution to identify new treatment targets and/or biomarkers for an early treatment intervention.

The method is described in detail in **Salmon et al., 2021**. Briefly, **CTCs** were enriched using **density-gradient centrifugation** and the mononuclear cell layer was incubated with MicroBeads targeting **Glycophorin-A** (CD235a) and **CD45** followed by two separations on LS Columnsl. The resulting cell suspension was fixed with 2% paraformaldehyde blocked with 3 %BSA. **Immunofluorescence staining** was performed with **anti-CD11b, anti-CD45, anti-ERCC1 and anti-CK**. Stained cell suspensions were analyzed within a maximum of four days, using the **DEPAr-ray™ NxT** (Menarini Silicon Biosystems, Bologna, Italy) and single cells were selected manually based on fluorescence labeling and morphology. The genetic material of the isolated single cells was amplified (**WGA**) and DNA quality was assessed for downstream analysis of **copy number variation (CNV)** using the lowpass next generation sequencing. Ampli1™ LowPass library preparation was performed by Menarini Silicon Biosystems using the Hamilton Microlab STARlet platform (Hamilton company) followed by lowpass whole genome sequencing on an Illumina NovaSeq™ platform.

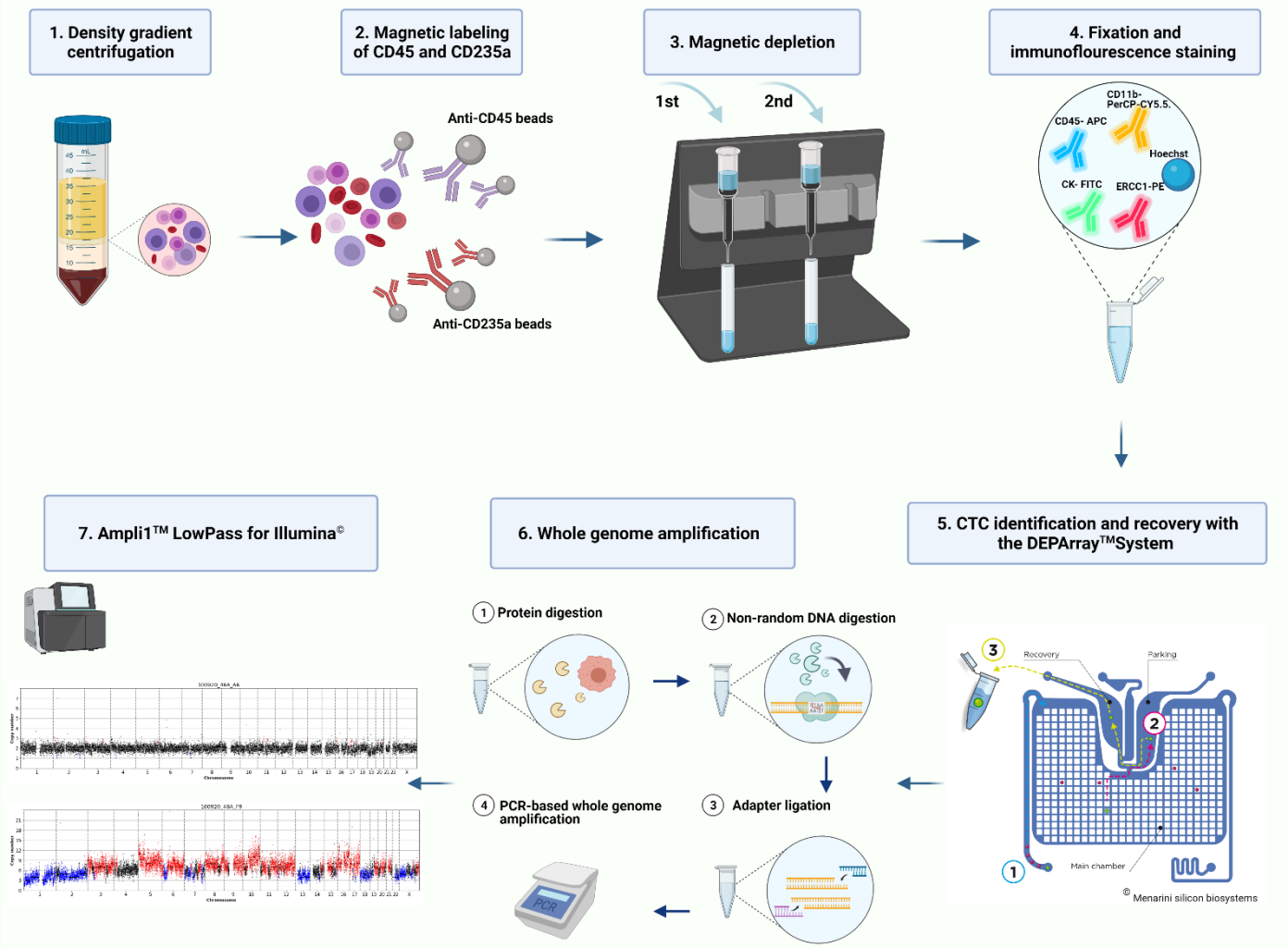

Figure 6. Workflow for single CTC isolation and molecular characterization in blood samples of OC patients. After density-gradient centrifugation and magnetic-based negative depletion of erythrocytes (CD235a) and leucocytes (CD45), immunofluorescence staining was performed. Subsequent single cell imaging and sorting using the DEPArray™ Nxt was followed by a whole genome amplification. After Ampli1™ low pass (Menarini Silicon Biosystems) library preparation, copy number variation sequencing was performed. Created with BioRender.com (***Salmon et al., 2021***).

We frequently detected the following **three cell types (Figure 7a-c)**:

Type A-cells: epithelial sCTC): Hoechst^{pos}, ERCC1^{pos}, CD45^{neg}, CD11b^{pos}, CK^{pos}.

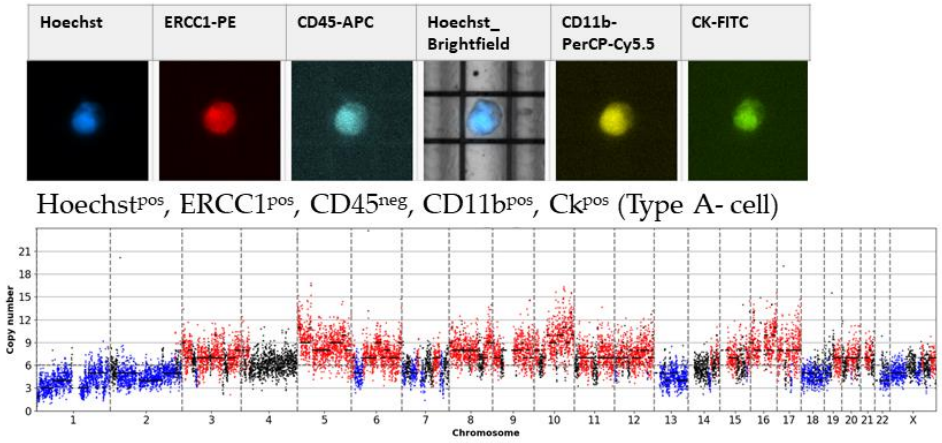

Figure 7a: Type A-cell, Hoechst^{pos}/ ERCC1^{pos}/CD45^{neg}/ CD11b^{pos}/CK^{pos}, and matched CN profile of Type A-cell with a ploidy of 6 (***Salmon et al., 2021***).

Type-B-cells: potential epithelial sCTC): Hoechst^{pos}, ERCC1^{pos}, CD45^{neg}, CD11b^{pos}, CK^{neg}.

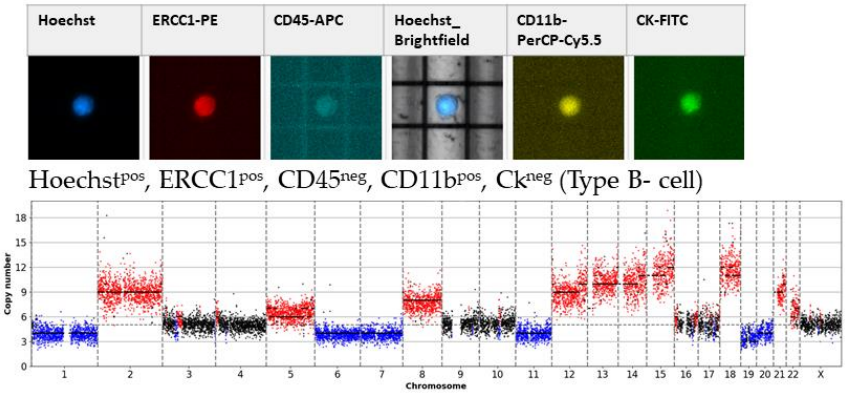

Figure 7b: Type B-cell, Hoechst^{pos}/ERCC1^{pos}/CD45^{neg}/CD11b^{pos}/CK^{neg} and matched CN profile of Type B- cell with a ploidy of 5 (***Salmon et al., 2021***).

Type C-cells: potential mesenchymal sCTC): Hoechst^{pos}, ERCC1^{pos}, CD45^{neg}, CD11b^{neg}, CK^{neg}.

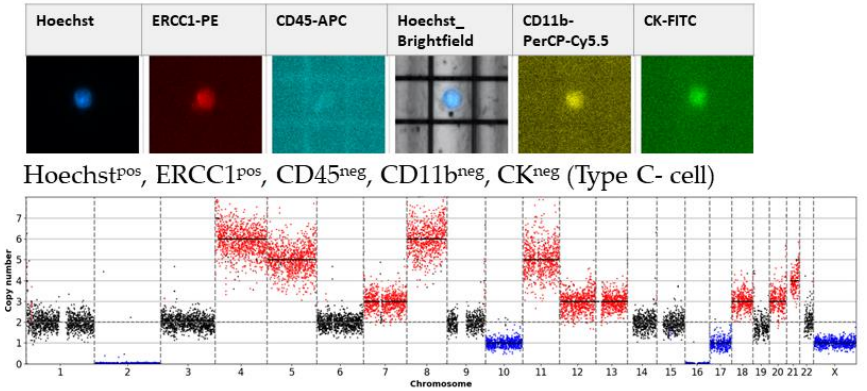

Figure 7c: Type C-cell, Hoechst^{pos}/ERCC1^{pos}/CD45^{neg}/ CD11b^{neg}/CK^{neg} and matched CN profile of Type C-cell with a ploidy of 2 (***Salmon et al., 2021***).

In 30.8% of **Type A- cells** (**Figure 8**)**,** their aberrant character was underlined through frequent genome wide Copy Number Alterations (**CNA**) with one representative profile illustrated in **Figure 7a**. In contrast, in **Type B-cells** as well as in **Type C-cells**, highly **altered CN** were only detected in **6.25%** and **4.76%** of **sCTCs**, respectively (**Figures 7b, 7c, 8**).

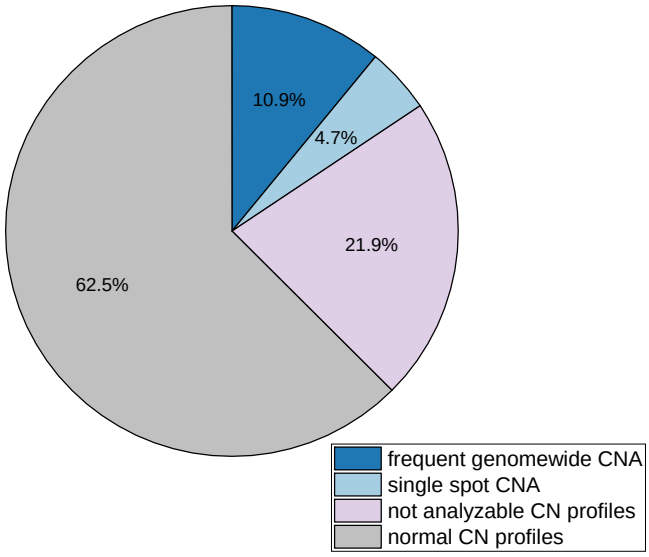

Figure 8: Copy number alteration frequency in all analyzed single cells (***Salmon et al., 2021***).

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